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(54) Title: A MONOCLONAL ANTIBODY TO HUMAN CARDIAC TROPONIN I

(57) Abstract

A monoclonal antibody having high affinity and specificity to cardiac troponin I is described. The antibody was shown to have an epitope within the cardiac specific region on the N-terminus of the protein. This monoclonal antibody is especially useful as a reagent in a rapid format immunoassay system to identify blood, serum or plasma levels of cardiac troponin I. Such an immunoassay system can be used for diagnosing and quantifying myocardial necrosis and infarction.

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A MONOCLONAL ANTIBODY TO HUMAN CARDIAC TROPONIN I

Field of Invention

This invention relates to a monoclonal antibody, which show specific binding to and high affinity for cardiac troponin I (cTnI). More specifically, this invention relates to the hybridoma cell line, designated as 31-265, and the monoclonal antibody produced by the same. The monoclonal antibody of the present invention can be used for determining blood, serum and plasma levels of cardiac troponin I. The antibody is particularly useful for rapid format diagnostic tests for cardiac muscle damage.

Background and Prior Art

Troponin is a thin-filament associated complex of the myocyte. The complex regulates calcium dependant interaction of myosin and actin, and thus controls muscle contraction. Troponin I is one of the three components in the complex which inhibits actin and myosin interaction in the absence of calcium (C.V. Perry, Biochem. Soc. Trans. 7, 593-617, 1979). There are three isoforms of TnI; slow skeletal TnI (ssTnI), fast skeletal TnI (fsTnI) and cardiac TnI (cTnI). The three isoforms of TnI show approximately 40% difference in amino acid sequence (J.M. Wilkinson, et al., Nature, 271, 31-35, 1978). The cardiac isoform contains a cardiac specific 31 amino acid N-terminal sequence (J.R. Vallins et al., FEBS Lett. 270, 57-61, 1990), as shown below:

H_2N-Ala-Asp-Gly-Ser-Ser-Asp-Ala-Ala-Arg-Glu-Pro-Arg-Pro-Ala-Pro-Ala-
Pro-Ile-Arg-Arg-Arg-Ser-Ser-Asn-Tyr-Arg-Ala-Tyr-Ala-Thr-Glu-OH

The predominant fetal troponin I isoform present in the heart is the ssTnI and complete transition to cTnI takes place in humans after birth (S. Sasse, et al., Circ. Res. 72, 9[illegible]-938, 1993). It is believed at present that the cTnI is the only TnI isoform expressed in the myocardium and it is not expressed in human regenerating skeletal muscles. This identifies cTnI as a valuable potential candidate for the biochemical diagnosis of myocardial injury.

The measurement of cTnI in serum using polyclonal rabbit antisera and its relevance as a biochemical marker for myocardial infarction (MI) was first described by Cummins (B. Cummins, et. al. Am. Heart. J. 113, 1333-1344, 1987). Using a radioimmunoassay (RIA), they found that cTnI concentration was elevated above normal levels 4 to 6 hours after the onset of MI, and remained elevated for up to 6 - 8 days.

In recent years, monoclonal antibodies specific against cTnI and non-reactive against skeletal TnI have been reported (G.S. Bodor, et. al., Clin. Chem. 38, 2203-2214, 1992; C. Larue, et. al., Mol. Immunol. 29, 271-278, 1992 and C. Larue, et al., Clin.Chem. 39, 972-979, 1993). By pairing cardiac-specific monoclonal antibodies, an enzyme-linked immunoassay (ELISA) has been developed for measurement of cTnI in human serum. Both groups describe cardiac isoform specificity of their monoclonal antibodies, by showing lack of cross reactivities with skeletal isoforms of TnI using sandwich ELISA. Bodor and coworkers further confirmed the specificity of their antibodies by immunoblotting with myofibrillar proteins. However, neither Larue's nor Bodor's group clearly demonstrate their monoclonal specificity for cTnI.

There remains a need for a human troponin I monoclonal antibody that demonstrates high affinity and specificity for cardiac troponin I to be used as a reagent in an immunoassay system. Such an immunoassay system, can be used for diagnosing and quantifying myocardial necrosis and infarction according to the rapid format procedure disclosed in U.S. Patent 5,290,678.

<u>Summary of Invention</u>

The limitations of the prior art are addressed in the present invention by providing a monoclonal antibody that is specific for and has high affinity for the cardiac isoform of troponin I. The monoclonal antibody of the present invention is also sensitive enough and has a high enough affinity to be useful as a reagent in an immunoassay system to identify cardiac troponin I in

the blood, serum or plasma of patients with cardiac muscle damage (e.g. myocardial infarction and unstable angina).

According to the present invention there is provided a novel murine hybridoma which secretes a monoclonal antibody of defined specificity against and high affinity for human cTnI.

In one embodiment of the present invention there is provided a monoclonal antibody specific for cTnI produced by a hybridoma cell line which is referred to as 3I-265, deposited with American Type Culture Collection on August 25, 1994 under Accession Number HB 11710. The monoclonal antibody from this cell line has shown cTnI specificity as determined by ELISA, BIAcore biosensor analysis and Western blot, when tested against three isoforms of TnI and also ten other cardiac myocyte proteins. The specificity was further confirmed by a lack of cross reactivity with human serum albumin or human IgG. The monoclonal antibody of this embodiment of the present invention was shown to recognize and have high affinity for an epitope within the cardiac specific region of the N-terminus of the protein.

According to a further embodiment of the present invention, there is provided a method of detecting cardiac troponin I in a sample using a monoclonal antibody produced from hybridoma cell line 3I-265, deposited with American Type Culture Collection under Accession Number HB 11710, which comprises contacting the sample with the monoclonal antibody to effect an immunoreaction between the cardiac troponin I in the sample and the monoclonal antibody; and detecting the immunoreaction.

Brief Description of the Drawings

Fig. 1. Competitive ELISA on adsorbed human cardiac troponin I: binding of soluble peptide (residues 26 - 35 of cTnI) to MAb 3I-265 with competitive ELISA using adsorbed cTnI.

Fig. 2. Sandwich assay of cardiac troponin I : MAb 3I-265 was used as capture antibody and rabbit anti-cTnI as detector.

Fig. 3. Western blotting of human TnI isoforms against anti TnI monoclonals and isoelectric focusing (IEF).

(A) Coomassie blue-stained SDS-PAGE (reduced)

Lane 1 : Molecular weight standards

Lane 2 : Human cTnI

Lane 3 : Human fast skeletal TnI

Lane 4 : Human slow skeletal TnI

(B) Western blot of (A) with MAb 3I-265

(C) Coomassie blue/crocein scarlet-stained isoelectric focusing on Bio-Rad IEF media 3 - 10 of purified anti-TnI monoclonals

Lane 1 & 6 : pI standards

Lane 2 : MAb 3I-265

Detailed Description of the Invention

The monoclonal antibody of the present invention can be distinguished from the antibodies known in the art in terms of its diagnostic value due to its specificity, sensitivity and high affinity for cardiac troponin I.

The monoclonal antibody of the present invention was prepared by conventional procedures, generally following the methods of Kohlers and Milstein (Nature, 256, 495-497, 1975; Eur. J. Immunol. 6, 511-519, 1976). According to this method, tissue culture adapted mouse myeloma cells are fused to antibody producing cells from immunized mice to obtain hybrid cells that produce large amounts of a single antibody molecule. In general the antibody producing cells are prepared by immunizing an animal, for example, mouse, rat, rabbit, sheep, horse, or bovine, with an antigen. The immunization schedule and the concentration of the antigen in suspension is such as to provide useful quantities of suitably primed antibody producing cells. These antibody producing

cells can be either spleen cells, thymocytes, lymph node cells and/or peripheral blood lymphocytes.

The antibody producing cells are then fused with myeloma cells, cell lines originating from various animals such as mice, rats, rabbits, and humans, can be used, using a suitable fusion promoter. Many mouse myeloma cell lines are known and available generally from members of the academic community and various depositories, such as the American Type Culture Collection, Rockville, Maryland. The myeloma cell line used should preferably be medium sensitive so that unfused myeloma cells will not survive in a selective media, while hybrids will survive. The cell line most commonly used is an 8-azaguanine resistant cell line, which lacks the enzyme hypoxanthine-guanine-phosphoribosyl-transferase and therefore will not be supported by HAT (hypoxanthine-aminopterin-thymidine) medium. In general, the cell line is also preferably a "non-secretor" type, in that it does not produce any antibody. The preferred fusion promoter is polyethyleneglycol having an average molecular weight from about 1000 to about 4000. Other fusion promoters such as polyvinylalcohol, a virus or an electrical field can also be used.

The immortalized cells (hybridoma) must then be screened for those which secrete antibody of the correct specificity. The initial screening is generally carried out using an enzyme-linked immunosorbent assay (ELISA). Specifically, the hybridoma culture supernatants are added to microtitre plates which have been previously coated with the antigen, in this case human cardiac troponin I. A bound specific antibody from the culture supernatants can be detected using a labelled second antibody, for example, goat antimouse IgG labelled with peroxidase. Cultures that are positive against the antigen are then subjected to cloning by the limiting dilution method. Secondary hybridoma cultures are re-screened as described above, and further positive cultures are then examined using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden). The cultures are then evaluated as to determine whether or not the antibody binds the antigen and to determine the kinetic profile of antigen

binding. Selected cultures based on these results are subject to further cloning until culture stability and clonality are obtained. Immediately after hybridization, the fusion products will have approximately 80 chromosomes, and as these cells proceed to divide they will randomly lose some of these chromosomes. The cloning process is to select those cells which still have the chromosomes coding for antibody production. The cloning process is repeated until 100 % of the sub-population exhibits the production of a specific antibody, which is indicative of the "stability" of the hybridoma. In addition, hybridoma culture wells often have multiple colonies some of which may be antibody non-producers. The cloning process allows the selection of a positive hybrid which is derived from a single cell.

The monoclonal antibody of the present invention can be produced either using a bioreactor or from ascites, both procedures of which are well known in the art.

The monoclonal antibody of the present invention can be used in an immunoassay system for determining blood, serum or plasma levels of cardiac troponin I.

Current immunoassays utilize a double antibody method for detecting the presence of an analyte. These techniques are reviewed in "Basic Principals of Antigen-Antibody Reaction", Elvin A. Labat, (Methods in Enzymology, 70, 3-70, 1980). Such systems are often referred to as fast format systems because they are adapted to rapid determinations of the presence of an analyte. The system requires high affinity between the antibody and the analyte. According to one embodiment of the present invention, the presence of cardiac troponin I is determined using a pair of antibodies, each specific for troponin I and at least one antibody specific for cardiac troponin I. One of said pairs of antibodies is referred to herein as a "detector antibody" and the other of said pair of antibodies is referred to herein as a "capture antibody". The monoclonal antibody of the present invention can be used as either a capture antibody or a

detector antibody. The monoclonal antibody of the present invention can also be used as both capture and detector antibody, together in a single assay. One embodiment of the present invention thus uses the double antibody sandwich method for detecting cTnI in a sample of biological fluid. In this method, the analyte (cTnI) is sandwiched between the detector antibody and the capture antibody, the capture antibody being irreversibly immobilized onto a solid support. The detector antibody would contain a detectable label, in order to identify the presence of the antibody-analyte sandwich and thus the presence of the analyte.

Common early forms of solid supports were plates, tubes or beads of polystyrene which are well known in the field of radioimmunoassay and enzyme immunoassay. More recently, a number of porous material such as nylon, nitrocellulose, cellulose acetate, glass fibres and other porous polymers have been employed as solid supports.

One embodiment of the present invention uses a flow-through type immunoassay device. Valkirs et al. (U.S. Patent No. 4,632,901) discloses a device comprising an antibody, specific to an antigen analyte, bound to a porous membrane or filter to which is added a liquid sample. As the liquid flows through the membrane, target analytes bind to the antibody. The addition of the sample is followed by the addition of a labelled antibody. The visual detection of the labelled antibody provides an indication of the presence of the target analyte in the sample.

Another example of a flow-through device is disclosed in Kromer et al. (EP-A 0 229 359), which described a reagent delivery system comprising a matrix saturated with a reagent or components thereof dispersed in a water soluble polymer for controlling the dissolution rate of the reagent for delivery to a reaction matrix positioned below the matrix.

In migration type assays, a membrane is impregnated with the reagents needed to perform the assay. An analyte detection zone is provided in which labelled analyte is bound and assay indicia is read. For example, see Tom et al. (U.S. Patent 4,366,241), and Zuk (EP-A 0 143 574). Migration assay devices usually incorporate within them reagents which have been attached to coloured labels thereby permitting visible detection of the assay results without the addition of further substances. See for example Bernstein (U.S. Patent 4,770,853), May et al. (WO 88/08534), and Ching et al. (EP-A 0 299 428). The monoclonal antibody of the present invention can be used in all of these known types of flow-through devices.

Direct labels are one example of labels which can be used according to the present invention. A direct label has been defined as an entity, which in its natural state, is readily visible, either to the naked eye, or with the aid of an optical filter and/or applied stimulation, e.g. U.V. light to promote fluorescence. Among examples of coloured labels, which can be used according to the present invention, include metallic sol particles, for example, gold sol particles such as those described by Leuvering (U.S. Patent 4,313,734); dye sole particles such as described by Gribnau et al. (U.S. Patent 4,373,932) and May et al. (WO 88/08534); dyed latex such as described by May, supra, Snyder (EP-A 0 280 559 and 0 281 327); or dyes encapsulated in liposomes as described by Campbell et al. (U.S. Patent 4,703,017). Other direct labels include a radionucleotide, a fluorescent moiety or a luminescent moiety. In addition to these direct labelling devices, indirect labels comprising enzymes can also be used according to the present invention. Various types of enzyme linked immunoassays are well known in the art, for example, alkaline phosphatase and horseradish peroxidase, lysozyme, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, urease, these and others have been discussed in detail by Eva Engvall in Enzyme Immunoassay ELISA and EMIT in *Methods in Enzymology*, 70. 419-439, 1980 and in U.S. Patent 4,857,453.

Other examples of biological diagnostic devices, which can be used for the detection of cTnI, using the monoclonal antibody of the present invention, include the devices described by G. Grenner, P.B. Diagnostics Systems, Inc., in U.S. Patents 4,906,439 and 4,918,025.

In one embodiment of the present invention, the diagnostic test uses a blood sample tube which is commonly used to draw blood samples from patients. The inside wall of the tube acts as a carrier for the monoclonal or polyclonal antibodies and required reagents or detection means, needed to produce a measurable signal. In this embodiment the capture antibody is immobilized onto the wall of the test tube. After the sample is drawn from the patient, the user simply shakes the sample with the detector antibody in the tube so that the detector antibody reacts with any cTnI in the blood. In this example the monoclonal antibody of the present invention can be either the capture antibody or the detector antibody. It may be necessary to use a sample wherein the red blood cells have been removed, so that the red blood cells will not interfere with the analysis of the results. If the analyte is present in the blood, it will be sandwiched between the capture antibody and the detector antibody which contains a suitable label for direct detection or reacts with the reagents in an indirect assay. The solid support (the test tube) can then be rinsed free of unbound labelled material. A variety of solid supports can be used according to this method, for example, test tube walls, plastic cups, beads, plastic balls and cylinders including microtitre plates, paper, and glass fibres.

There are currently available several types of automated assay apparatus which can undertake rapid format assays on a number of samples contemporaneously. These automated assay apparatus include continuous/random access assay apparatus. Examples of such systems include OPUS™ of PB Diagnostic System, Inc. and the IMX™ Analyzer introduced by Abbott Laboratories of North Chicago, Illinois in 1988. In general, a sample of the test fluid is typically provided in a sample cup and all the process steps including pipetting of the sample into the assay test element, incubation and

reading of the signal obtained are carried out automatically. The automated assay systems generally include a series of work stations each of which performs one of the steps in the test procedure. The assay element may be transported from one work station to the next by various means such as a carousel or movable rack to enable the test steps to be accomplished sequentially. The assay elements may also include reservoirs for storing reagents, mixing fluids, diluting samples, etc. The assay elements also include an opening to permit administration of a predetermined amount of a sample fluid, and if necessary, any other required reagent to a porous member. The sample element may also include a window to allow a signal obtained as a result of the process steps, typically a fluorescent or a colorimetric change in the reagents present on the porous member to be read, such as by means of a spectroscopy or fluorometer which are included within the assay system.

The automated assay instruments of PB Diagnostic Systems, Inc. are described in U.S. Patents 5,051,237; 5,138,868; 5,141,871 and 5,147,609.

A description of the IMX Analyzer is included in the "Abbott IMX Automated Bench Top Immunochemistry Analyzer System" by Fiore, M. et al., *Clinical Chemistry*, 35, No. 9, 1988. A further example of these analyzers has been described in U.S. Patent 4,956,148 entitled "Locking Rack and Disposable Sample Cartridge" issued to C.J. Grandone on September 1, 1990, and assigned to Abbott Laboratories, which describes a carousel for carrying a plurality of reaction cells for use in connection with the Abbott IMX™ system. A further development in the art has been described in Canadian Patent Application 2,069,531, Chadwick M. Dunn et al., assigned to Abbott Laboratories wherein the immunochemistry analyzer system, described in this prior art application, has the capability of testing for up to three or four analytes in a single batch during a single run using currently available instrumentation. The system described in the Canadian application referred to above enables the users to group three small batches of assays together rather than run three separate analysis. The

monoclonal antibody of the present invention can be used in these automated analyzers.

A further class of immunochemical analyzer systems, in which the monoclonal antibody of the present invention can be used, are the biosensors or optical immunosensor systems. In general an optical biosensor is a device which uses optical principles quantitatively to convert chemical or biochemical concentrations or activities of interest into electrical signals. These systems can be grouped into four major categories: reflection techniques; surface plasmon resonance; fibre optic techniques and integrated optic devices. Reflection techniques include ellipsometry, multiple integral reflection spectroscopy, and fluorescent capillary fill devices. Fibre-optic techniques include evanescent field fluorescence, optical fibre capillary tube, and fibre optic fluorescence sensors. Integrated optic devices include planer evanescent field fluorescence, input grading coupler immunosensor, Mach-Zehnder interferometer, Hartman interferometer and difference interfermoter sensors. These examples of optical immunosensors are described in general in a review article by G.A. Robins (Advances in Biosensors), Vol. 1, pp. 229-256, 1991. More specific description of these devices are found for example in U.S. Patents 4,810,658; 4,978,503; 5,186,897; R.A. Brady et al. (Phil. Trans. R. Soc. Land. B 316, 143-160, 1987) and G.A. Robinson et al. (in Sensors and Actuators, Elsevier, 1992).

In one embodiment of the present invention, cTnI is detected in a sample of blood, serum or plasma, using the monoclonal antibody of the present invention, in a device comprising a filter membrane or solid support with a detection section and a capture section. The detector section contains an antibody (a detector antibody), which will react with the cTnI. The detector antibody is reversibly immobilized onto the solid support and will migrate with the sample, when in use. It is preferred that the detector antibody is labelled, for example with a radionucleotide, an enzyme, a fluorescent moiety, luminescent moiety or a coloured label such as those described in the prior art, and discussed above. The capture section comprises a capture antibody, which is irreversibly

immobilized onto the solid support. The antibodies, capture and detector antibody, and the necessary reagents are immobilized onto the solid support using standard art recognized techniques, as disclosed in the flow-through type immunoassay devices discussed previously. In general, the antibodies are absorbed onto the solid supports as a result of hydrophobic interactions between non-polar protein substructures and non-polar support matrix material.

According to this embodiment of the present invention, if the cTnI is present in the sample, it will react with the detector antibody in the detector section and will migrate onto the filter membrane towards the capture section where the analyte will further bind with the capture antibody. Thus, the cTnI will be sandwiched between the capture antibody and the detector antibody, which contains a suitable label.

In this example of the present invention, if the detector antibody is labelled with a coloured label or an enzyme which will produce a coloured label, the patient's blood would first require centrifugation or some pre-filtering in order to remove the red blood cells so that the colour of the red blood cells will not interfere with the coloured labels. If radioactive labels or florescent labels are to be used, a pre-filtration or centrifugation step may not be required. In this embodiment, the monoclonal antibody of the present invention can be either the capture antibody or the detector antibody. In one embodiment, the monoclonal antibody of the present invention is a capture antibody. The detector antibody can be other cardiac specific cTnI antibodies, monoclonal antibodies reactive to other isoforms of troponin I, or polyclonal anti-troponin I antibodies. Either chicken, rabbit, goat or mouse polyclonal antibodies can be used. Many such antibodies are known and can be prepared and labelled by known methods.

This immunoassay system is generally described in U.S. Patent 5,290,678. The antibody of this invention is particularly useful in this system because of its high affinity and specificity for cardiac troponin I.

The following detailed examples will further illustrate the invention, which are not to be construed as limiting.

Examples

Example 1: Preparation of Monoclonal Antibody Against Human cTnI

(1) Immunization

Balb/c mice, a strain with H-2^d haplotype from Charles River Canada, St. Constant, Quebec, Canada, female, 7 - 9 week old, were immunized with purified human cTnI. Primary injection was given with a total of 25 μg - 100 μg antigen by two routes, one intravenously (i.v.) in 20 mM Tris HCl, pH 8.5, 0.5M NaCl, 60 mM 2-mercaptoethanol, and the other subcutaneously (s.c.) with complete Freund's adjuvant. Subsequent immunizations of 3 - 6 times were carried out at intervals of 3 - 4 weeks by intraperitoneal (i.p.) inoculation of 25 μg - 100 μg antigen with incomplete Freund's adjuvant. Immunized mice were sacrificed 3 - 4 days after the final immunization given either i.v. or i.p. in the same 20 mM Tris buffer.

(2) Myeloma cells

Sp2/0 mouse myeloma cells were obtained from ATCC (ATCC CRL-1581).

(3) Preparation of Hybridoma

Immunocytes, prepared from the spleen of mice immunized with cTnI, and the myeloma cells, both as described above, were fused in the presence of polyethylene glycol (PEG) according to the method described by Fuller, S.A., Takahashi, M., and Hurrell, J.G.R., (Preparation of Monoclonal Antibodies: In: Ausubel F, Brent B, Kingston R., et. al., eds. Current Protocols in Molecular Biology. New York: Greene Publishing Associates, 1987: Unit 11). The resulting fused cells were suspended in the HAT selection medium and plated onto 96-well plates which were pre-seeded with feeder cells, PEC (peritoneal exudate cells), as described by Fuller et al. (see above reference).

Fresh HAT medium was added on day 7, and on day 9, 50% of the culture medium was removed and replaced with fresh HAT medium.

Culture supernatants were first screened for the presence of specific antibodies by solid-phase ELISA. The antigen was immobilized directly onto the plastic surface of a 96-well Immunolon-4, flat-bottom microtiter plates (Dynatech Labs, Chantilly, VA) by incubating overnight at 4°C with 100 µl per well of protein solution at 1 µg/ml in 100 mM carbonate buffer, pH 9.6. The excess binding sites were blocked by bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.2. After washing the plate with PBS containing 0.05% Tween 20, 100 µl of the culture supernatants containing the monoclonal antibodies were incubated with the immobilized antigen for 1h at 37°C. After washing, peroxidase conjugated goat anti-mouse IgG (Jackson ImmunoResearch Lab, Inc., West Grove Penn.) was added and incubated for 30 min. at 37°C. After the last washing, orthophenylene diamine (OPD) (Sigma Chemicals, St. Louis Missouri), 10 mg in 12.5 ml 0.1 mol/L citrate buffer, pH 5.0, containing 125 µl 3% H_2SO_4 was added and optical density was read at 490_{nm}. Positive cultures were fed with fresh medium and 24 hours later, ELISA screening was repeated, as described above. Cultures giving the same or greater OD signal to the first ELISA were transferred onto 24-well culture plates pre-seeded with feeder cells, as described above.

Positive hybridoma cultures were cloned by the limiting dilution method, as described by Fuller et al. (see above reference). The secondary cultures were again screened by solid-phase ELISA against cTnI, as described above. Positive wells for anti-cTnI were then further screened for cross reactivity with fsTnI and ssTnI, using solid phase ELISA, according to the method described above.

Cultures producing cTnI-specific antibodies were expanded onto 24-well culture plates. When expanded cultures were confluent, the cTnI-specificity of the antibodies in the culture supernatants were further examined

using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden). The system uses surface plasmon resonance, which detects changes in optical properties at the surface of a thin gold film on a glass support. Detailed theoretical background and procedures are described by R. Karlsson, et. al. (J. Immunol. Methods, 145, 229, 1991).

Monoclonal antibodies at a constant concentration of 30 μg/ml in 10 mM Hepes, 0.15 M NaCl, 3.4 mM ethylenediaminetetraacetic acid disodium salt, 0.05 % surfactant 20 (HBS, pH 7.4) were allowed to interact with sensor surfaces on which rabbit anti-mouse IgG_{Fc} (obtained from Jackson ImmunoResearch Lab, Inc., West Grove, Penn.) had been immobilized. The antigen, cTnI, at concentrations ranging from 1.25 μg/ml to 20 μg/ml, was allowed to interact with the bound monoclonal antibodies. The runs were performed at 25°C, at a flow rate of 5 μl/min during 6 min. (30 μl injection). After the run, the surface was regenerated by injecting a 1 M formic acid solution during 1 min. (5 μl injection). The BIAcore system analysis confirms not only the cTnI-specificity of the antibody but also the capability of the same to capture cTnI in solution. The latter can be a critical confirmation of the usefulness of the antibody. Often clones screened and isolated by solid-phase ELISA fail to recognize the antigen in solution. Such antibodies can not be utilized in diagnostic immunoassay systems.

A selected culture, based on the results obtained by solid-phase ELISA and BIAcore analysis, was further subjected to cloning until culture stability and clonality were obtained. This hybridoma cell line was deposited with the American Type Culture Collection on August 25, 1994 under Accession Number HB 11710.

(4) Production of Monoclonal Antibodies

Balb/c mice, as described above, previously treated with 0.5 ml of pristane were injected intraperitoneally with 1 - 5 x 10^6 cloned hybridoma cells in 0.5 ml phosphate buffered saline (PBS), pH 7.4. Approximately 2 weeks later,

ascites were collected and the monoclonal antibody was affinity purified n a Protein A or Protein G column, using known procedures. The purified monoclonal antibody was used for various immunochemical studies.

Example 2: Specificity of the 3I-265 Monoclonal Antibody Against Human cTnI

(1) Solid-phase ELISA

Specificity test of the TnI monoclonal antibody of the present invention against various cardiac myocyte proteins as well as human serum albumin and human IgG was performed by solid-phase ELISA (see Table 1). All of the purified proteins were immobilized directly onto the plastic surface of a flat-bottom microtitre plate (Dynatech Labs, Chantilly VA) by incubating overnight at 4°C with 100 μl per well of protein solution at 1 μg/ml in 100 mM carbonate buffer, pH 9.6. The excess binding sites were blocked by bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.2. After washing the plate with PBS containing 0.05% Tween 20, 100 μl of the monoclonal antibody at 10 μg/ml was added for 1h at 37°C. After washing, peroxidase conjugated goat anti-mouse IgG (Jackson ImmunoResearch Lab, Inc., West Grove Penn.) was added and incubated for 30 min. at 37°C. After the last washing, orthophenylene diamine (OPD) (Sigma Chemicals, St. Louis Missouri), 10 mg in 12.5 ml 0.1 mol/L citrate buffer, pH 5.0, containing 125 μl 3% H_2SO_4 was added and optical density was read at 490_{nm}.

As shown in Table 1, the 3I-265 monoclonal antibody showed no cross-reactivity with any of the various analytes, as tested by solid-phase ELISA, with the exception of troponin I. Monoclonal antibody 3I-265 was specific for the cardiac isoform of TnI.

Table 1 Specificity Test of the TnI Monoclonals

	31-265
Cardiac Troponin-I	++++
Slow Skeletal Troponin-I	-
Fast Skeletal Troponin-I	-
Cardiac Troponin-T	-
Cardiac Troponin-C	-
Tropomyosin	-
CK-MB	-
CK-BB	
CK-MM	-
Myoglobin	-
Cardiac MLC1	-
Cardiac MLC2	-
Myosin Heavy Chain	-
Human Serum Albumin	-
Human IgG	-

O.D. 490 nm:	-	<0.09
	+	0.1-0.5
	++	0.5-1.0
	+++	1.0-1.5
	++++	>1.5

(2) Competitive ELISA

Binding of soluble peptide (residues 25 - 34 of cTnI) to MAb 3I-265 (cTnI specific MAb) with competitive ELISA using adsorbed cTnI was determined. MAb 3I-265 binding on adsorbed cTnI was inhibited by the synthetic peptide by 50% at 30 μg/ml. The first seven amino acid residues of the peptide are located within the 31 residues of the N-terminus on cTnI, which are not present on the skeletal isoforms. The result of this competitive ELISA indicates that the epitope of MAb 3I-265 resides within the cardiac specific region on the N-terminus of the protein.

(3) Sandwich ELISA

Cardiac TnI specific MAb 3I-265 was used as capture antibody (immobilized antibody) and rabbit anti-cTnI as detector antibody (labelled antibody). The rabbit antibody is a polyclonal antibody produced using known procedures. From the total polyclonal antibody preparation a cardiac specific fraction was isolated by affinity purification, using well known techniques. The MAb 3I-265 was immobilized onto microtitre plates using known procedures. Cardiac TnI and skeletal TnI were measured at concentrations ranging from 0.2 to 820 ng/ml in PBS, pH 7.2 containing 0.05% Tween 20 (TTBS). These standard TnI solutions were incubated with the immobilized antibody for 60 min at 37°C followed by, after washing with TTBS, 30 min incubation at 37°C with horse radish peroxidase (HRP)-labelled rabbit anti-TnI. This is a polyclonal antibody prepared using standard procedures against cardiac troponin I. The antibody preparation is conjugated with horse radish peroxidase, again using standard procedures. After washing, enzyme activity was measured by addition of OPD substrate solution, as described above. After incubation for 30 min in the dark, the reaction was stopped with 2M H_2SO_4. The absorbance was read at 490_{nm}.

As shown in Fig. 2, the minimum detectable level of cTnI in the sandwich ELISA was 0.5 ng/ml, while skeletal TnI was not detected at 800 ng/ml.

Example 3: Physico-chemical characterization of the 3I-265 anti-cTnI monoclonal antibody and antigenic specificity

(1) Antibody Class and Subclass

Antibody class and subclass were determined by ELISA with a commercial kit (Bio-Rad, Hercules California, Cat. no. 172-2055), using the method described by the manufacture. As shown in Table 2, MAb 3I-265 is a IgGl,k.

(2) Isoelectric Point (pI value)

Isoelectric focusing of the anti-TnI monoclonal antibody 3I-265 was performed using the Model 111 Mini IEF cell (Bio-Rad, Hercules California Cat. no. 1702975) following the instructions provided by the manufacturer. The pI value of MAb 3I-265 is 7.2, as shown in Figure 3C.

(3) Affinity constants

Kinetic and affinity constants for the interaction between the 3I-265 monoclonal antibody and cTnI were determined using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden). The system uses surface plasmon resonance, which detects changes in optical properties at the surface of a thin gold film on a glass support. Detailed theoretical background and procedures are described by R. Karlsson, et. al. (J. Immunol. Methods, 145, 229, 1991).

Kinetic runs were performed as follows: The monoclonal antibody, at a constant concentration of 30 μg/ml in 10 mM Hepes, 0.15 M NaCl, 3.4 mM ethylenediaminetetraacetic acid disodium salt, 0.05 % surfactant 20 (HBS, pH 7.4), was allowed to interact with sensor surfaces on which rabbit anti-mouse IgG_{Fc} (Jackson ImmunoResearch Lab, Inc.) had been

imm bilized. The antigen, cTnI, at concentrations ranging from 1.25 μg/ml to 20 μg/ml, was allowed to interact with the bound monoclonal antibody. The run was performed at 25°C, at a flow rate of 5 μl/min during 6 min. (30 μl injection), taking a total of 24 report points. After injection of the antigen was complete, dissociation of the antigen from the antibody was monitored by taking a total of 18 report points. After the run, the surface was regenerated by injecting a 1 M formic acid solution during 1 min. (5 μl injection). The instrument software produces a table of dR_A/dt and R_A values that can be directly used in a plotting program (Microsoft Excel).

As shown in Table 2, the Kd(M) value of MAb 3I-265 is 3.9 x 10^{-8}. It is worth noting that the log affinity constant value estimated on BIAcore appear 1.0 lower than the actual value.

(4) <u>Antigenic specificity determined by Western blot</u>

The antigenic specificity of the 3I-265 monoclonal antibody was determined by immunoblotting assay. Purified human TnI, either cardiac or skeletal isoforms, was electrophoresed on polyacrylamide gel in sodium dodecyl sulphate (SDS-PAGE) and transferred onto nitrocellulose membrane. These methods are well known in the art, as described for example in Tsang, V.C.W. et al. Methods in Enzymol. Vol. 92, 377, 1983. The nonspecific binding sites on the blots were blocked with 5% skim milk solution in TTBS buffer followed by incubation with TTBS buffer, pH 7.5, containing purified monoclonal anti-TnI at 1 to 10 μg/ml (listed in Table 2) for 1 h. Blots were washed with TTBS buffer, pH 7.5, and incubated further with goat anti-mouse IgG labelled with horseradish peroxidase (HRP) (Bio-Rad, Hercules California, Cat no. 170-6516). Colour was developed using 4-chloro-1-naphthol, as described previously. The colour development was stopped by washing in distilled water. The results obtained are shown in Figure 3A and 3B and summarized in Table 2. Monoclonal antibody 3I-265 was specific for cTnI.

Table 2 Physico-chemical Characterization and Antigenic Specificity of anti-TnI Monoclonals

MAb	Isotype	Western	pI	Kd(M)
3I-265	IgG1,k	cTnI	7.2	3.9×10^{-8}

Example 4: Detection of Cardiac Troponin I in a Biological Sample

In this example, the monoclonal antibody produced from hybridoma cell line 3I-265 was used as a capture antibody in a flow through assay system, based on the double antibody sandwich assay.

A sample of a patient's serum (50 μl to 150 μl) was added to the assay system through a sample opening, which was in fluid communication with a reagent pad containing a labelled detector antibody. The detector antibody was an affinity purified cTnI polyclonal antibody prepared from troponin I-immunized rabbits, as described in a preceding example. If the sample size was small a carrier fluid was added after the application of the sample. The carrier fluid can be any buffer solution; for example phosphate buffer, saline, Tris-HCl or water. If the sample contained cTnI it will bind to the detector antibody in the reagent pad. The detector antibody was reversibly immobilized and thus migratible with the sample. The sample continued to flow from the reagent pad onto a filter membrane, onto which the monoclonal antibody of the present invention was irreversible immobilized (capture antibody). Labelled detector antibody-cTnI complex, if present, will bind to the capture antibody on the filter membrane. The presence of the analyte, which has been labelled with the labelled detector antibody, will thus be positioned at the location of the capture antibody, which generally coincides in position to a display window in the assay system.

All references cited herein are specifically incorporated by reference.

Although the disclosure describes and illustrates preferred embodiments of the invention, it is to be understood that the invention is not limited to these particular embodiments. Many variations and modifications will now occur to those skilled in the art. For a definition of the invention, reference is made to the appended claims.

We claim:

1. Hybridoma cell line 31-265, deposited with American Type Culture Collection under Accession Number HB 11710.

2. Monoclonal antibody produced from hybridoma cell line 31-265, deposited with American Type Culture Collection under Accession Number HB 11710.

3. A method of detecting cardiac troponin I in a sample using a monoclonal antibody produced from hybridoma cell line 31-265, deposited with American Type Culture Collection under Accession Number HB 11710, which comprises contacting the sample with the monoclonal antibody to effect an immunoreaction between the cardiac troponin I in the sample and the monoclonal antibody and detecting the immunoreaction.

FIG. 1

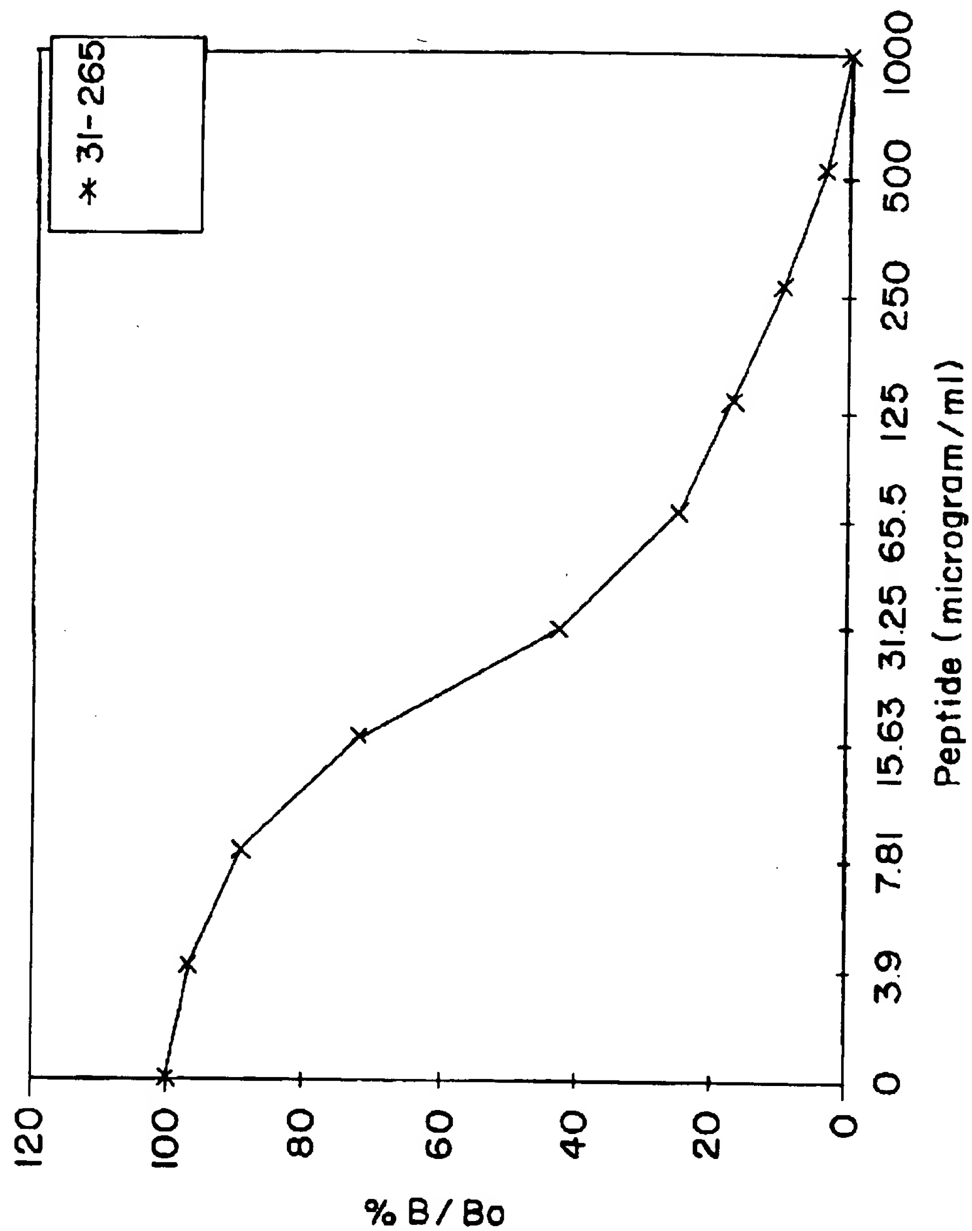
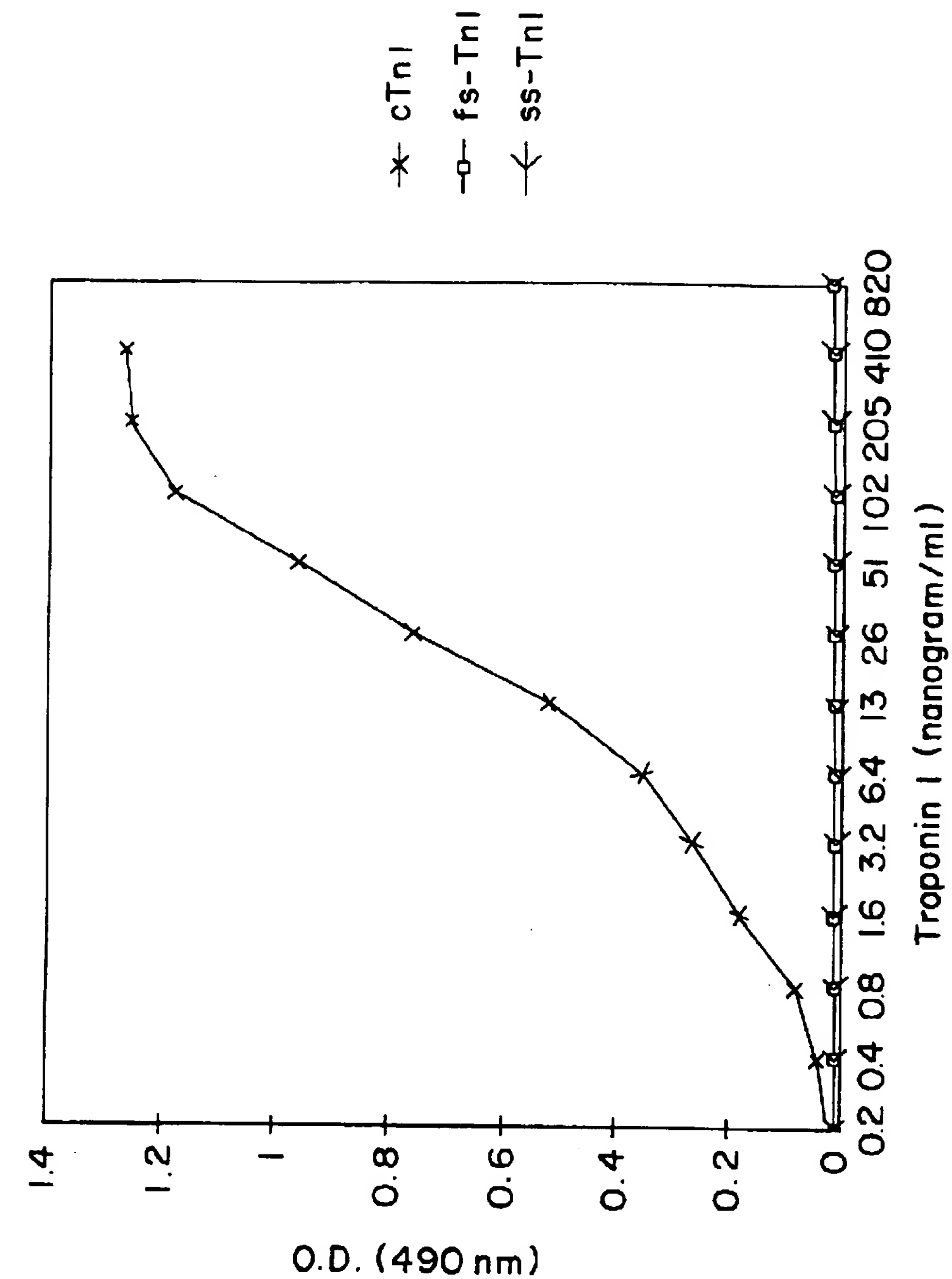

* 31-265
% B / Bo
120
100
80
60
40
20
0
Peptide (microgram/ml)
0
3.9
7.81
15.63
31.25
65.5
125
250
500
1000

FIG. 2


cTnI
fs-TnI
ss-TnI
O.D. (490 nm)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Troponin I (nanogram/ml)
0.2 0.4 0.8 1.6 3.2 6.4 13 26 51 102 205 410 820

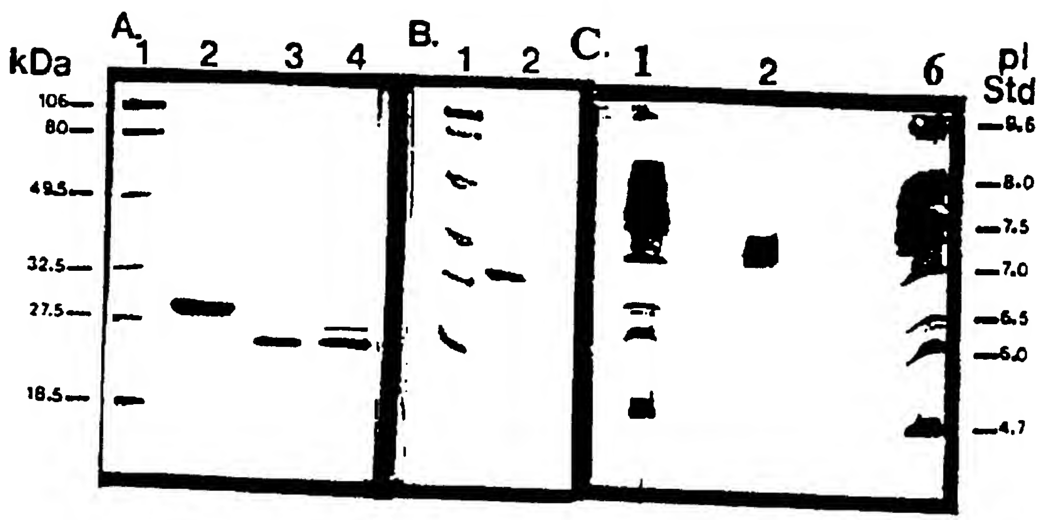

kDa
A. 1 2 3 4
B. 1 2
C. 1 2 6
pI Std
106
80
49.5
32.5
27.5
18.5
9.6
8.0
7.5
7.0
6.5
6.0
4.7

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 95/00804

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/20 C07K16/18 G01N33/577 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOLECULAR IMMUNOLOGY, vol. 29, no. 2, February 1992 OXFORD, GB, pages 271-278, C. LARUE ET AL. 'New monoclonal antibodies as probes for human cardiac troponin I: Epitopic analysis with synthetic peptides.' see the whole document ---	1-3
X	DE,A,42 43 648 (BOEHRINGER MANNHEIM GMBH) 7 July 1994 see the whole document ---	1-3
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 October 1995	14. 12. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CLINICAL CHEMISTRY, vol. 38, no. 11, November 1992 WASHINGTON, DC, USA, pages 2203-2214, G. BODOR ET AL. 'Development of monoclonal antibodies for an assay of cardiac troponin-I and preliminary results in suspected cases of myocardial infarction.' see the whole document ---	1-3
X	CLINICAL CHEMISTRY, vol. 36, no. 6, June 1990 WASHINGTON, DC, USA, page 1103 G. BODOR ET AL. 'Human cardiac troponin I measurement in suspected myocardial infarction with a double monoclonal antibody sandwich ELISA.' see abstract 0706 ---	1-3
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X	CLINICAL CHEMISTRY, vol. 40, no. 6, June 1994 WASHINGTON, DC, USA, page 1018 C. LARUE ET AL. 'Rapid and specific immunoenzymometric assay of cardiac troponin I for myocardial infarction diagnosis.' see abstract 0155 ---	1-3
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Int ...tional Application No
PCT/IB 95/00804

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB,A,2 248 688 (G. JACKOWSKI) 15 April 1992 cited in the application see the whole document ---	1-3
P,X	DISEASE MARKERS, vol. 12, no. 3, 1995 CHICHESTER, GB, pages 187-197, K. HAIDER ET AL. 'Production and characterisation of anti-cardiac troponin-I monoclonal antibodies.' see abstract -----	1-3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 95/00804

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		EP-A-	0664002	26-07-95
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		AU-B-	8475791	16-04-92
		BE-A-	1004994	16-03-93
		CN-A-	1060533	22-04-92
		DE-A-	4122886	16-04-92
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		IT-B-	1251682	19-05-95
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